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(54) Title: METHODS FOR PRODUCING DAIRY PRODUCTS, IN PARTICULAR CHEESE USING LACTIC ACID BACTERIA
PROVIDED WITH ADDITIONAL NEUTRAL PROTEASE ACTIVITY

(57) Abstract

The present invention relates to a method for carrying out a process of biotransformation of a substrate whereby at least one lactic
acid bacterium comprising a gene encoding a neutral protease having an altered resulting activity, e.g. a neutral protease having an altered
stability and/or specificity, is used. Further, a food product, e.g. a cheese, obtainable by the said method is disclosed.

Title:  Methods for producing dairy products, in particular
        cheese using lactic acid bacteria provided with
        additional neutral protease activity.


        The invention relates to processes for making fermented
food products from raw starting materials such as milk,
meat, cereals and vegetables.
        Fermenting these materials is often carried out using
5    so called lactic acid bacteria. Lacic acid bacteria include
members of the genera *Lactobacillus*, *Lactococcus*,
*Leuconostoc*, *Pediococcus* and *Streptococcus*. The fermenting
step is carried out for a number of reasons. It helps to
enhance the time over which the food product can be
10   preserved and it can add to the development of flavour and
texture of the product. In addition, some fermented milk
products are stated to have health and nutritional benefits.
        The invention especially relates to processes for
making cheese.
15       Processes for making cheese are of course different for
each different kind of cheese to be made. In principle
however there are a number of main steps in the process
which are essentially the same.
        First of all to the milk (be it full milk, or skimmed
20   milk, pasteurized ot not) a starter culture, rennet and
usually calcium are added to start coagulation.
After an appropriate time interval, during which the
coagulum is cut and stirred, allowing for sufficient
syneresis the whey is drained off.
25       The remaining material is called the curd. The curd has
to ripen into cheese, but this is where the processes start
to vary, depending on the kind of cheese to be made.
In the case of gouda type cheese, the curd is pressed in a
cheese vat (or mould) and then transferred to the brine. The
30   cheeses are left to soak in the brine for a time interval in

the order of days and are then transferred to the warehouse for further ripening.

Thus from the moment that the cheese is pressed there is hardly any possibility to add anything to the cheese.

5   Cheddar type cheeses are brought into contact with the salt before pressing, during milling of the curd. Thus for cheddar cheese it is possible to add substances until a later moment in the cheese making process. The lactic acid bacteria are usually present in the starting culture. Lactic

10   acid bacteria convert lactose into lactic acid, but they also produce proteolytic enzymes that degrade proteins present in the milk. The activity of the lactic acid bacteria is present throughout the whole process. The proteolytic activity especially, is very important for the

15   ripening of the cheese. It determines for a large part the taste and texture characteristics of the resulting cheese. Therefore it is desirable to be able to design the rate of ripening and the extent of ripening that a cheese will be subjected to.

20   It has been suggested to enhance the rate of ripening by adding proteolytic enzymes during various stages of the process. When enzymes are added to the milk, only a small fraction thereof is retained in the curd (El Soda, 1990). This means that a relatively high amount of expensive enzyme

25   preparations have to be added. Moreover, even in processes where the enzymes may be added later on, for instance to the curd, it will be hard, if not impossible to ensure an even distribution of the enzyme through the curd.

A way to provide for a more even distribution of

30   materials (especially brine) through the curd has been suggested in EP-A-0 492 716. Herein injection of liquids under high pressure into the curd or cheese are disclosed. This process seems rather complicated and expensive and rather unsuitable for enzymes or microorganisms, which are

35   likely to degrade under high pressure, because of for instance shear forces.

It remains a desire, for many reasons disclosed herein, to be able to provide the curd with additonal enzyme activity which is evenly distributed through the curd. Additions of *B.subtilis* neutral protease preparations were shown to contribute in a significant amount to the degradation of proteins present in cows or ewe's milk during ripening of cheddar cheese (Fernandez-Garcia et al.,1990). However, as stated before, an equal distribution of enzyme through the curd, and thus an equal rate of ripening is very hard to obtain in that manner.

A possible way to ensure equal distribution of the ripening proteolytic activity through the curd, is by providing the lactic acid bacteria of the starter culture with the wanted proteolytic activity. This can be accomplished by introducing the gene encoding the enzyme of interest in said lactic acid bacteria. Using a heterologous gene expression vector van der Guchte et al. (1989, 1990, 1991, 1992a, 1992b) obtained expression of several heterologous genes in *L. lactis,* including the *Bacillus subtilis* neutral protease gene and the gene of hen egg white lysozyme. The contribution of the *B. subtilis npr* gene product, as expressed by *L. lactis,* to the ripening and flavor development of Cheddar cheese was tested by McCarry et al. (1994). Their results showed that cheeses produced with *L. lactis* harboring the *B. subtilis npr* gene, on the expression vector constructed by van der Guchte et al. (1991) were subject to greatly accelerated proteolysis, resulting in an overripening of the cheeses within one month.

The invention provides a method for carrying out a process of biotransformation of a substrate whereby at least one lactic acid bacterium comprising a gene encoding a neutral protease having an altered activity is used. For instance by using a lactic acid bacterium which has a neutral protease gene of more limited stability, especially in processes of making cheese, it is possible to get enhanced ripening of the cheese, but to limit said

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enhancement in time and rate. Instead of altering the
duration of the activity of the neutral protease, it is of
course also very suitable to provide a neutral protease
which has a lower activity for a longer period of time, or
5    to regulate the expression of the neutral protease so that
the activity can be switched on or off more or less at will.

It is also very useful to provide neutral proteases
with altered specificity, as also disclosed in the present
invention. The important aspect is that bacteria are
10   provided which have a neutral protease activity which is
especially designed for their application. Therefore
combinations of mutations in neutral protease genes are
preferred embodiments of the present invention. For
instance, it may be very useful to have a highly active
15   neutral protease with a very limited stability, or a mildly
active neutral protease with prolonged stability, or vice
versa depending on the specific application. All altered
neutral protease activity and/or specificity and/or
stability are preferably under control of an inducible
20   mechanism for expression, preferably an inducible promoter.
Especially preferred is a system whereby the neutral
protease has different activities/stabilities, preferably
under control of a regulatable mechanism, are used or are
available for use when necessary. By having an array of
25   different stabilities of neutral protease activity
available, it is possible to regulate and monitor the cheese
ripening process, or other proteolytic biotransformation
processes for that matter, in detail. Therewith the taste
characteristics of the cheese can also be influenced with
30   the required subtileness. A class of organisms well known
for their neutral proteases is the genus *Bacillus*. For this
genus a number of neutral proteases and their variants have
been disclosed. Especially *Bacillus subtilis* proteases are
suitable for the present invention. It will be clear that
35   for many applications according to the invention it may not
be necessary to provide the lactic acid bacterium with a
complete gene encoding the relevant protease. Parts thereof

resulting in a polypeptide having the same kind of activity may of course be used. These parts may even be specifically chosen for reasons of a different stability or half-life. Variants of the exemplified proteases are also part of the
5  invention. They may be also specifically chosen for their different properties in terms of stability, activity and half-life. Some preferred mutations of Bacillus subtilis proteases include at least one of the following

: mutation of the codon encoding leucine at
10  position 300;

: deletion of the codon encoding leucine at position 300;

: mutation of the codons for residues 120, 134 and/or 136;

15      : mutation of the codons 186 and 194 to cysteine residues; or

: mutation of the codons 102 and 120 to cysteine residues.

Suitable expression and/or other regulatory elements
20  should be provided with the gene to be expressed by the lactic acid bacteria. Suitable elements for regulated expression of the neutral proteases according to the invention include, but are not limited to the prtP promoter, which can be induced by specific dipeptides; the nisin
25  promoter, which can be induced by nisin; the superoxide dismutase promoter, which can be induced by oxygen; the LacZ promoter, inducible by IPTG; and T7 polymerase, inducible by lactose. These elements are well-known in the art. Preferred are regulatory elements which are derived from lactic acid
30  bacteria themselves, because they are usually best suited for expression in those bacteria. Most preferred are autologous regulatory elements, such as the prtP, the nisin and the SOD promoter. For transferring the gene and the regulatory elements into the host cell many vehicles are now
35  known. These vehicles may all be used according to the present invention. Preferred are those transfer vehicles which are designed to integrate the relevant gene into the

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host genome, without integration of any material from the transferring vehicle.

Recently developed procedures have made it possible to insert genes of interest into the chromosome of *L. lactis* at

5   desired positions, and in such a way that, apart from the heterologous gene, no additional DNA sequences are incorporated. As a result, very well defined genetically modified strains can be obtained, that are equipped with additional genetic information, resulting in a micro-

10  organism with improved enzymatic properties. Although these procedures were initially developed for use in *L. lactis*, they are applicable to other lactic acid bacteria and other microorganisms as well. Examples thereof are known and given herein below. The choice of the host cell to be provided

15  with the additional protease activity depends on the kind of process of biotransformation to be carried out. For making cheese it should be a lactic acid bacterium which can be added to or is part of the starter culture. A very suitable genus of lactic acid bacteria is the *Lactococcus* genus.

20      It is possible according to the invention to produce an array of different lactic acid bacteria having additional protease activity, all of which may have different activities, stabilities and/or half-lifes. It is of course also possible to provide a single lactic acid bacterium with

25  different protease activities. By providing such a set of different protease activities, it is possible to fine tune the ripening process of cheese or to fine tune other biotransformation processes. Therefor it is possible to obtain for instance cheeses within a shortened ripening time

30  interval, but it is also possible to obtain cheeses with different taste characteristics. These products which were not obtainable until the present invention are also part thereof.

The invention will now be explained in more detail in

35  the following detailed description.

Detailed description.

The neutral proteases (NP's) constitute a group of
extracellular metallo-endopeptidases that are produced by
5    several members of the genus *Bacillus*. Representatives of
this class are used in several industrial processes, the
most important of which is the preparation of the artificial
sweetener aspartame (Gerhartz, 1990; Isowa *et al.*, 1979).
NP's are also employed in the leather and baking industry,
10   in breweries, and in the production of protein hydrolysates
(Gerhartz, 1990). At present, thermolysin (e.g. in the
preparation of aspartame) and the neutral protease of
*Bacillus subtilis* (e.g. for beer-brewing applications) are
the most frequently used NP's in industrial processes.
15   Several Bacilli are known to produce NP's. These enzymes
contain 300-319 amino acid residues and are active in the
neutral pH range. The best known is thermolysin, the highly
thermostable 316-residue NP from *B. thermoproteolyticus*.
*Bacilli* exhibit considerable differences in optimal growth
20   temperatures and the thermostabilities of their neutral
proteases differ accordingly. Several *Bacillus* neutral
proteases have been characterized and genes coding for these
enzymes have been cloned and sequenced from e.g. *B. subtilis*
(Yang *et al.*, 1984), *B. stearothermophilus* CU-21 (Fujii *et*
25   *al.*, 1983; Takagi *et al.*, 1985), *B. stearothermophilus* MK-
232 (Kubo & Imanaka, 1988), *B. thermoproteolyticus rokko*
(Marquardt *et al.*, 1990), and *B. caldolyticus* (Van den Burg
*et al.*, 1991). Alignment of the amino acid sequences of the
NP's shows a high level of sequence similarity between the
30   different representatives (Figure 1.a). Using genetic
techniques (such as 'site directed mutagenesis', e.g.
Stanssens *et al.*, 1989), genes encoding NP's have been
mutated to change properties of the enzymes, such as thermal
stability, activity and specificity (Imanaka *et al.*, 1986;
35   Toma *et al.*, 1991; van den Burg *et al*, 1991; Eijsink *et al.*,
1991; Kubo *et al.*, 1992). These experiments have led to a
better understanding of the mechanisms that influence

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thermostability of NP's. Application of this knowledge has
resulted in the construction of mutant NP's, characterized
by thermostabilities exceeding that of any of the known NP's
(Eijsink et al., 1995). Additionally mutant NP's have been
5    constructed that are characterized by a decreased
thermostability. Availability of thermostable variants is
desirable, since it expands the temperature range at which
NP-catalysed processes can be conducted and enzyme activity
lasts longer, reducing the amount of enzyme required.
10   Variants with reduced thermostabilities can be useful in
applications where enzymatic activity is only required
during the initial stages of the process, or where it can be
stopped by a simple heat treatment. Thermostability of NP's
is, at least under experimental conditions, determined by
15   the rate at which local unfolding processes occur, rendering
the molecules susceptible to autolysis (Eijsink et al.,
1991c, 1992c; Vriend and Eijsink, 1993; Dahlquist et al.,
1976; Braxton and Wells, 1992). As a consequence,
thermostable variants are more resistant towards
20   autoproteolysis, whereas thermolabile NP's are more
vulnerable towards autoproteolytic breakdown.
     The three dimensional structure of thermolysin has been
solved at 2.3 Å (Figure 2.a; Matthews et al., 1972a, b;
Colman et al., 1972). The enzyme contains one zinc ion and
25   four calcium ions, which are needed for activity and
stability, respectively. The molecule is generally
considered to consist of two domains, interconnected by a
central α-helix (residues 137-150). The C-terminal domain
may be divided into two subdomains connected by the 235-247
30   α-helix (Fontana, 1988; Eijsink et al., 1992). In the active
site cleft (indicated by an arrow in Figure 2.a) the zinc
ion is bound. It is coordinated by the side chains of
Glu166, His142, His146, and a water molecule, and these
residues are conserved in all Bacillus NP's. The zinc ion
35   and the fully conserved Glu143, His231 and, to a lesser
extent, Tyr157 play an important role in the catalytic
mechanism (Kester and Matthews, 1977; Hangauer et al , 1984;

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Tronrud *et al* ., 1986). The catalytic mechanism of
thermolysin is well established owing to extensive
crystallographic studies of complexes between this enzyme
and specific inhibitors (Hangauer *et al.*, 1984; Tronrud *et*
5   *al.*, 1986; Toma *et al* ., 1989; Dunn, 1989) . The
conservation among the NP' s of those residues that are
involved in catalysis in thermolysin suggests that all
neutral proteases have the same catalytic mechanism. This
supposition is strongly supported by crystallographic
10  analysis of the *B. cereus* NP (Pauptit *et al.*, 1988) which
showed an arrangement of presumed active site residues
similar to that in thermolysin. Site-directed mutagenesis
experiments have confirmed the importance of the residues
Glu143 and His231 for catalysis in the *B. subtilis* NP.
15  During catalysis the zinc coordinated water molecule is
forced in the direction of the Glu143 side chain by the
incoming substrate. As a result, the nucleophility of the
water molecule is enhanced resulting in a nucleophilic
attack on the carbonyl carbon of the scisile bond, resulting
20  in cleavage. Residue His231 is important in stabilizing the
transition state of the proteolytic reaction. Specificity
and activity of the enzyme is additionally determined by
amino acid residues in the substrate binding sites $S_1$, $S_2$,
$S_1$' and $S_2$' (Hangauer *et al.*, 1984; Matthews *et al.*, 1988).
25  Substrate specificities of NP's are, furthermore, determined
by residues constituting the entrance of the active site
cleft. The presence of specific large and/or charged
residues at particular positions can restrict the sizes of
the substrates still able to enter. Thus, substitution of
30  specific residues constituting the active site entrance
(e.g. residue 120 in *B. subtilis* NP) can increase the number
of substrates that can reach the active site.
      The present invention includes the use of variants of
the *B. subtilis* neutral protease displaying altered
35  activities and specificities, as obtained by site-directed
mutagenesis of specific residues involved in the interaction
between the enzyme and substrates (Gln120 and Phe134).

Mutations affecting the so-called hinge bending of the NP's
are also included. Hinge bending, the motion of the N-
terminal (residues 1-150) and C-terminal domains of NP
relative to each other, can be manipulated by mutations in
5    the interconnecting α-helix (137-150), or by amino acid
replacements influencing the non-covalent interactions
present between the two domains. Evidence for a role of
hinge bending in substrate specificity has come from the
comparison of the 3-dimensional structures of thermolysin
10   and *B. cereus* NP (Pauptit *et al., 1988; Stark et al., 1992).
The individual domains of both NP's were highly
superimposible with only minor differences in the backbone
tracing. However, the relative positions of the domains
deviated several degrees. This difference can contribute to
15   the somewhat different substrate specificities of the two
NP's. In fact, mutants of the *B. subtilis* and
*B. stearothermophilus* NP's that were constructed to effect
the hinge bending, by substituting Gly residues in the
interconnecting α-helix (in particular residue 136 in
20   *B. subtilis* NP), were shown to behave differently on
synthetic substrates, when compared to their wild-type
counterparts (O.R. Veltman, unpublished results).
        Using site directed mutagenesis several structural
elements in B. *subtilis* NP have been analysed with respect
25   to their impact on thermostability. An example of a residue
that affects stability is the C-terminal amino acid Leu300.
Analysis of the 3-dimensional model of the NP (Figure 2.b)
suggested that this particular residue is involved in the
stabilization of interactions between the two sub domains in
30   the C-terminal domain by occupying a hydrophobic pocket
formed by residues from both subdomains. Indeed, deletion of
Leu300 or substitution of this amino acid by a smaller
(Ala), a polar (Asn), a sterically unfavourable (Ile), or
deletion of Leu300, gave rise to NP's with decreased
35   thermostabilities (Table 1; Eijsink *et al.*, 1990) . It has
been shown that thermostability of NP's can be affected by
the introduction of disulfide bridges (Table 1; van den Burg

et al., 1992) In general, disulfide bridges are considered to contribute to stability by decreasing the entropy of the unfolded state. Several examples of the construction of active enzymes with correctly formed disulfide bonds exist.

5  The stability of the mutant enzymes often decreased, due to conformational strain and the effect of the amino acid replacement as such (Wetzel et al., 1987; Alber et al., 1989; Pjura et al., 1990) leading to irreversible inactivation at elevated temperatures (Wetzel et al., 1987;

10  Mitchinson and Wells, 1989; van den Burg et al., 1992). In B. subtilis NP disulfide bridges have been introduced by replacing amino acid residues Val102 and Gln120, and Glu186 and Ala194, by cysteines. These positions were selected on the basis of positions of autodigestion target sites

15  determined previously (van den Burg et al., 1990). It was anticipated that disulfide bonds close to sites prone to autoproteolysis would make these regions less susceptible by rigidification of local structural elements. However, autodigestion of the enzymes was not notably affected by the

20  newly introduced Cys residues. On the other hand, thermal stability of the variants had decreased dramatically. Structural analyses of the model indicated that the destabilizing effects were caused by replacement of the individual side chains of residues 102, 120, 186 and 194 by

25  cysteine, giving rise to unfavourable local interactions. In particular amino acid Glu186 has an important role in NP with respect to thermal stability as it is involved in $Ca^{2+}$ binding. Calcium ions have been reported to be involved in maintaining the stability of a number of enzymes, e.g.

30  trypsin, $\alpha$-amylase, glutamine synthetase (Feder et al., 1971). NP's contain 2-4 $Ca^{2+}$ ions, and it has been shown that removal of calcium from thermolysin and B. subtilis NP is deleterious for stability (Grandi et al., 1980). The role of bound calcium ions was analysed in detail in

35  thermolysin by several authors (Drucker and Borchers, 1971; Roche and Voordouw, 1978; Corbett and Roche, 1983; Fontana et al., 1986). On the basis of biochemical data and analysis

of the thermolysin crystal structure, four calcium ions have
been identified in thermolysin (Drucker and Borchers, 1971).
Circular dichroism of calcium-loaded and calcium-depleted
thermolysin indicated conformational changes upon removal of

5    calcium. Fontana et al. (1986) showed that these
conformational changes correlated well with increased
autoproteolysis. The number of bound calcium ions varies
between the Bacillus NP's. The thermostable variants contain
four calcium ions, the thermolabile variants only two, a

10   difference which may underlie the difference in
thermostability. Transplantation of a calcium binding loop
from thermolysin to B. subtilis NP rendered the latter
enzyme more thermostable at high $Ca^{2+}$ concentrations (0.1 M).
At these concentrations the half-life of denaturation of the

15   mutant NP, as determined by circular dichroism, was
significantly higher. At low $Ca^{2+}$ concentrations (1 mM) no
effect on stability was observed (Toma et al., 1991).


**METHODS**

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*Plasmids and strains*
     The B. subtilis npr gene (originally obtained from
L. Mulleners, Gist-Brocades) was subcloned in the high copy
number plasmid pTZI2 (Aoki et al., 1987) , yielding pGS1

25   (Figure 3; Eijsink et al., 1990). The gene was expressed in
the protease deficient B. subtilis strain DB117 (Eijsink et
al., 1990). Suitable fragments of the npr gene were
subcloned in the E. coli plasmid pMa/c for site-directed
mutagenesis (Stanssens et al., 1989). E. coli WK6 and

30   WK6MutS (Zell and Fritz, 1987) were used in site-directed
mutagenesis procedures. All strains were grown on Trypton-
Yeast medium, containing the appropriate antibiotics.
     Neutral protease gene variants were cloned in plasmid
pORI28 (Figure 4). This plasmid is able to replicate in

35   specific E. coli, B. subtilis and L. lactis strains that
express the repA gene from their chromosomes. The npr gene
variants were subcloned using either specific restriction

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endonucleases or, alternatively, polymerase chain reaction-
based methods. The latter approach uses specific
oligonucleotides with sequence homology to the start and end
of the structural *npr* gene and additional restriction sites
5    for subcloning purposes. After subcloning of the *npr* genes
in pORI28, promoter sequences of lactococcal origin (Figure
5) were cloned upstream of the genes, resulting in secretion
of active protease by *L. lactis*. *L. lactis* strains were
grown in M17-based media. Protease producing colonies were
10   selected on agar plates supplemented with 0.8% skim milk by
their ability to degrade the substrate, which results in a
clearing zone around the colonies.

The DNA encompassing *npr* and the lactococcal promoter
was isolated from colonies expressing NP and subsequently
15   cloned in pINT51 (Figure 6). pINT51 contains a DNA fragment
derived from the chromosome of *L. lactis* LB250. In the
middle of this fragment several restriction endonuclease
recognition sites are available for insertion of the
*npr*/promoter combination. The plasmids thus obtained are not
20   able to replicate in *L. lactis* LB250, but are able to insert
into the genome of this strain by homologous recombination.
In case a single cross-over event occurs, the whole plasmid
becomes part of the lactococcal genome, which can be
selected for by the resistance of such strains to the
25   antibiotic erythromycin and to develop blue colonies in the
presence of the substrate X-Gal. To eliminate of the
antibiotic resistance marker and other plasmid DNA
sequences, with the exception of the npr gene, cells are
grown for several generations in the absence of
30   erythromycin, resulting in a second recombination event that
excises all original pINT51 sequences from the chromosome
(Figure 7). Cells in which this has taken place were
selected by their inability to become blue in the presence
of the chromogenic substrate X-Gal.

*Selection and construction of mutants*

The design of destabilizing or activity effecting
mutations, theoretical analysis of their structural effects,
and structural inspections of the neutral protease molecule
5  was performed using *in computo* procedures described elsewere
(Eijsink *et al,* 1990; Vriend and Eijsink, 1993). In these
procedures general accepted principles of protein structure
and stability were taken into account (e.g. Matthews *et al.,*
1990; Alber *et al.,* 1991; Vriend and Eijsink, 1993, Fersht
10  and Serrano, 1993).

In the mutagenesis procedure mutagenic oligonucleotides
used for the production of site-specific mutations were
designed such that restriction sites were removed or
created, without producing additional changes at the amino
15  acid level. Mutagenesis was performed using a gapped duplex
method described earlier (Stanssens *et al.,* 1989). Mutant
clones were selected by restriction analysis and their *npr*
gene fragments were sequenced using the dideoxy chain
termination method (Sanger *et al.,* 1977). For the production
20  of mutant neutral proteases in *B. subtilis,* correctly
mutated *npr* gene fragments were used to construct derivates
of pGS1 containing an intact *npr* gene.


*Production, purification and characterization of neutral*
25  *proteases*

*B. subtilis.*DB117 harbouring a pGS1 variant was
cultured in 600 ml medium in aerated 1000 ml flasks at 32°C.
After 16 hours of cultivation the cells were removed by
centrifugation and the supernatants were loaded onto
30  Bacitracin-silica columns (Unilever Research Laboratories,
Vlaardingen, The Netherlands) for affinity chromatography as
described previously (Van den Burg *et al.,* 1989) . After
purification the enzymes were stored at -18°C in the elution
buffer used in the affinity chromatography procedure (20 mM
35  sodium acetate, pH 5.3; 5 mM $CaCl_2$; 20% (v/v) isopropanol;
2.5 M NaCl; 0.3% sodium azide). Purified enzyme was analyzed
using sodium dodecyl sulfate polyacrylamide gel

electroforesis (SDS-PAGE) as described before (Van den Burg
et al., 1989).

Thermostability, expressed as T50, was determined by
incubating aliquots of diluted pure enzyme (approx. 0.1 µM
5   in 20 mM sodium acetate, pH 5.3; 5 mM $CaCl_2$; 0.5% (v/v)
isopropanol; 62.5 mM NaCl) at appropriate temperatures.
Subsequently, the residual protease activity was determined
using a casein assay (Fujii et al., 1983). The T50 and δT50
values presented in this invention are derived from at least
10   three independent measurements. The standard deviation in
T50 values is approximately 0.7°C; that in δT50 approximately
0.4°C.

Specific activities of wild-type and neutral proteases
variants were determined using casein, and the synthetic
15   substrates FAGLA (furylacryl and FAAFA (furylacryl). $K_m$ and
$k_{cat}$ values were determined using Lineweaver-Burke
methodology.

Substrate specificities of wild-type and neutral
protease variants were analyzed using β-casein as a
20   substrate. Neutral protease and β-casein were incubated at a
enzyme-substrate ratio of $10^{-3}$ (w:w) in 50 mM sodium acetate,
pH 7.5; 5 mM $CaCl_2$; 50 mM NaCl, and incubated for 16 hours
at 30°C. After incubation TFA (trifluoroacetic acid) was
added, to a final concentration of 1%, and the TFA-soluble
25   reaction products were separated from the TFA-insoluble
products by centrifugation at 13,000 x g. The supernatant
was removed and dried by evaporation. Dried peptide
preparations were stored at -18°C. Casein degradation was
analyzed using HPLC reversed phase chromatography on a
30   Biorad HPLC system. Peptides were separated on a reversed-
phase column (250 x 4.6 mm, Hi-Pore 318, Biorad). Solvent A
was 0.11% TFA (v/v) and 5% acetonitrile (v/v) in Milli Q
water, Solvent B was 0.1% TFA (v/v) and 60% acetonitrile
(v/v) in Milli Q water. TFA-soluble peptides were dissolved
35   in solvent A and applied to the column at a flow rate of
1 ml/min. Elution was performed at room temperature using a

linear gradient (0-80 %) of solvent B. Peptides were
detected by UV absorption at 214 nm.

*Characterization of L. lactis strains expressing neutral*
5   *protease variants*

L. lactis strains in which the wild-type *npr* gene with
different lactococcal promotor sequences or *npr* gene
variants with L. *lactis* promotor P32 had been stable
integrated, as described above, were analyzed with respect
10   to growth characteristics, neutral protease production, and
proper processing of the gene product.

Growth characteristics of L. *lactis* LB250 and variants
thereof expressing the *npr* gene were compared in M17-based
medium, supplemented with glucose, and in reconstituted skim
15   milk. Neutral protease production of the variants was
compared by determining neutral protease activity in culture
supernatants using casein as a substrate. Processing of the
neutral protease by LB250 variants was analyzed using anti-
neutral protease rabbit antibodies. To 1 ml of overnight
20   culture supernatant 10 µl TFA was added and TFA-insoluble
proteins were precipitated by centrifugation. The pellet was
dried and resuspended in SDS-PAGE loading buffer. Prior to
SDS-PAGE samples were boiled for 5 minutes. After
electrophoresis proteins were blotted to nitrocellulose
25   membranes and neutral protease was detected using anti-Npr
antibodies according to Towdin and Gordon (1984). Attempts
to detect intracellular unprocessed or processed forms of
the neutral protease after electrophoresis of cell-free
extracts of LB250 variants were hampered by cross-reactivity
30   with proteins from lactococcal origen.

Insertion of npr/promoter sequences in the L. *lactis*
genome was analyzed using Southern hybridization techniques,
essentially according to Maniatis (1982). Chromosomal DNA
was isolated from L. *lactis* and subsequently digested with
35   selected restriction endonucleases. After separation of the
resulting fragments by agarose gel electrophoresis, DNA was
blotted to nitrocellulose membranes. Recombination was

17

visualized using *npr* gene fragments as probes. Probes were
labelled using the ECL procedure (Amersham, Buckinghamshire,
UK). Detection was performed by a fluorescence technique
(ECL, Amersham, Buckinghamshire, UK), according to the
5    supplier. Parts of pINT51 were used as probes to ascertain
the removal of vector sequences from the *L. lactis* variants
genomes after the second recombination event.


EXAMPLE 1

10

*Construction of L. lactis variants expressing different*
*levels of the B. subtilis neutral protease*
        The *B. subtilis* neutral protease gene was amplified by
polymerase chain reaction (PCR) using the synthetic
15   oligonucleotides listed in Table 2 and plasmid pGS1 (Figure
3) as a template.
        The resulting 1700 bp DNA fragment contains the entire
*npr gene,* including the GTG start codon and the TAA
stopcodon. Upstream the startcondon an additional 16 bp
20   fragment is present, that contains the recognition sequences
for the restriction endonucleases MluI, SalI and PmlI.
Downstream the stop codon 16 bp are present that contain
recognition sequences for BamHI and SalI. After purification
of the PCR product the DNA fragment was digested with BamHI
25   and MluI and, subsequently, ligated in pORI28 (Figure 4)
that had been digested with the same restriction enzymes.
Next, the ligation mixture was transformed to *E. coli*
EC1000, a strain that contains a copy of the *repA* gene on
the chromosome, thus enabling replication of pORI28
30   derivates, and selected on TY plates supplemented with
100 µg/ml erythromycin. DNA from transformants was analyzed
by restriction analysis and transformants harbouring pCR10
(Figure 8; pORI28 with the *B. subtilis npr* gene) were
selected for further constructions. Due to the lack of
35   promoter sequences upstream the *npr* gene no protease
production was present in these transformants. To obtain NP
production lactococcal promoters were cloned upstream the

18

gene in pCR10. For this purpose three promoter sequences
were selected; P23, P32 and P44, respectively (Figure 5, van
der Vossen et al., 1987). Promoter sequences were obtained
by PCR, using the oligonucleotides listed in Table 2, and
5    pGKV223, pGKV232 and pGKV244 (van der Vossen et al., 1987).
Using the chloramphenicol gene as a reporter relative
activities were determined to be 3.2, 0.6 and 0.2 for P23,
P32 and P44, respectively. The 3'-oligonucleotides were
chosen such that the original ORF sequences downstream of
10   the promoters were removed after PCR. After amplification
and purification of the promoter fragments they were
inserted in pCR10, upstream the npr gene, yielding pCR12,
pCR13 and pCR14, resp (Figure 8). For that reason, pCR10 was
digested with PmlI and MluI. The P23 and P32 fragments were
15   digested with NsiI, made blunt with T4 DNA polymerase and
subsequently digested with MluI. The P44 fragment was
digested with HindIII, treated with T4 DNA polymerase and
thereafter digested with MluI. The different fragments were
purified, ligated and electrotransformed to L. lactis 108
20   (repA⁺). The transformation mixture was plated on M17 agar,
supplemented with 0.5% glucose, 1% succrose, 5 µg/ml
erythromycin, and 0.8% skim milk. The latter addition
facilitates the detection of protease producing colonies, by
the ability of the protease to digest milk proteins,
25   resulting in a clear zone around the colony. Protease-
positive transformants were selected and the plasmid DNA was
analyzed by restriction analysis. From pCR12, pCR13 and
pCR14 promoter/npr fragments were isolated after digestion
with NotI and PstI. These fragments were ligated with pINT51
30   digested with the same restriction enzymes, yielding pCR112,
pCR113 and pCR114 (Figure 8). After electrotransformation
these constructs were selected for on M17 agar as described
above, additionally supplemented with chromogenic substrate
X-gal (0.006%). DNA from protease proficient blue colonies
35   was further analyzed by restriction analysis. DNA from
pCR112, pCR113 and pCR114 was electrotransformed to
L. lactis LB250, followed by selection on M17 plates

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containing erythromycin, skim milk and X-gal. Due to the fact that L. lactis LB250 does not contain the repA gene, erythromycin-resistant, halo-forming and blue-colouring colonies must be the result of a recombination event, in
5    particular a single cross-over (Figure 7).

This recombination event was analyzed by Southern hybridization. Total chromosomal DNA from positive clones was digested and after electrophoresis transferred to nitrocellulose. Presence and orientation of pCR112, pCR113
10   and pCR114 DNA was determined using the npr gene or parts of pINT51 as probes. Clones in which a proper recombination had taken place were subsequently grown for approximately 100 generations without selecting for erythromycin resistance. This results in loss of part or all of the integrated
15   sequences by a second recombination event. Ideally, the pINT51 sequences are lost, whereas the promoter/npr sequences retain in the chromosome. This situation can be selected for by screening for white colonies (due to the loss of the lacZ gene) that are still able to form halo's.
20   Several of the clones thus obtained were characterized in detail by Southern hybridization. Hybridization with npr as a probe yielded a clear signal (Figure 10), whereas a probe from pINT51 was not able to hybridize. Strains, positive with respect to these analyses, were designated LB212, LB213
25   and LB214 for containing the npr gene preceded by P23, P32 and P44 respectively. Since these strain do not contain any vector sequences, nor antibiotic resistance genes, they can be considered as "food-grade".

30   Characterization of L. lactis LB212, LB213 and LB214

Strains in which the B. subtilis npr gene had been integrated were analyzed with respect to growth and protease processing and production. Growth characteristics in M17-based medium were shown to be similar for the wild-type
35   LB250 and the three modified strains tested (Figure 11). Growth in milk

20

Protease production was determined on M17 plates
supplemented with skim milk. Whereas LB250 is not able to
form halo's on such plates, LB212, LB213 and LB214 colonies
were able to degrade the milk in the plates, resulting in
5    halo formation. The size of these halo's can be correlated
to the production levels of the protease. Comparison of the
halo sizes suggested similar production levels in LB212 and
LB213, whereas production by LB214 is significantly lower.
        Protease production was analyzed in culture supernatants
10   also. For that purpose, 150 µl from the supernatants of
early stationary phase cultures were incubated with 250 µl
of azocasein in 50 mM Tris-HCl, pH 7.5; 5 mM $CaCl_2$, 0.08%
$NaN_3$. After incubation for 19 hours at 37°C the reaction was
stopped by addition of 1.25 ml 10% TCA. After precipitation
15   of the acid-insoluble fraction, 0.9 ml of the supernatant
was added to 1.05 ml 1M NaOH and the absorbance at 440 nm
was measured. The amount of peptides released is a measure
of the proteolytic activity of the samples. The protease
activities of the different strains are listed in Table 3,
20   and show that the amounts of protease secreted by LB212 and
LB213 are similar, and several times higher than that of
LB214. The amounts of the strains LB282, LB292 and LB297 are
about half of that of LB212 and LB213.
        Proteins from overnight cultures (2 ml) were
25   precipitated by addition of TFA to a final concentration of
1% (v/v). Precipitated proteins were collected by
centrifugation, dried and dissolved in 50 µl SDS-PAGE
loading buffer. 20 µl was used for SDS-PAGE and after
electrophoresis the proteins were electrotransferred to
30   nitrocellulose membranes. Using anti-NP rabbit serum bands
migrating at the position of the mature form of the NP, as
produced by *B. subtilis,* were detectable in the samples
derived from LB212 and LB213. In the sample from LB214 no
signal was detectable, which might be caused by the low
35   production levels of the latter strain.

21

EXAMPLE 2

*Construction of L. lactis strains expressing B. subtilis neutral protease variants with decreased stabilities.*

B. *subtilis* neutral protease variants that show decreased thermal stabilities were selected to be integrated in the genome of L. *lactis*. These variants were previously constructed and tested in B. *subtilis*. Analyses of the variants showed that thermostability had decreased dramatically as the result of mutation the C-terminal residue Leu300 or by introducing disulfides between residues 102-120 and 186-194 (Table 1).

The genes encoding the NP variants were amplified by PCR using the oligonucleotides listed in Table 2. For the residue 300 variants the wild-type *npr* gene was the template and oligonucleotides introducing the substitutions or deletion were used in combination with oligonucleotide NP-5'. For the amplification of the disulfide variants, the genes already containing these changes, as present in B. *subtilis,* were used as templates for oligonucleotides NP-5' and NP-3'. The residue 300 variant PCR products were made blunt with T4 DNA polymerase and subsequently digested with MluI. The resulting fragment was ligated with pORI28 digested with EcoRV and MluI. The disulfide variants PCR products were digested with BamHI and MluI and ligated with pORI28 that had been digested with the same enzymes. After selection of the proper constructs, P32 was inserted in these constructs as described above. Using this strategy the following constructs were obtained: pCR22 (Leu300Ala); pCR32 (Leu300Ile); pCR42 (Leu300Asn); pCR52 (Leu300del); pCR62 (Val102Cys-Gln120Cys); pCR72 (Glu186Cys-Ala194Cys).

The constructs mentioned above gave rise to production of extracellular protease in L. *lactis* 108, as visualized by halo formation on plates supplemented with skim milk, except for the constructs pCR52 and pCR72. For the latter two mutants it is known that expression of these mutants in

*B. subtilis is* decreased also (B. van den Burg, V.G.H.
Eijsink, unpublished results).

    After transfer of the *npr* gene variants to pINT51, the
genes can be transferred to *L. lactis* LB250. After correct
5  and stable integration protease activities of the different
constructs can be compared. Strains with desirable
expression levels can subsequently be used in cheese
manufacturing experiments.


10  EXAMPLE 3


*Construction of L. lactis strain expressing B. subtilis*
*neutral protease variants with altered specificity*
    The use of *B. subtilis* NP variants as expressed by
15  *L. lactis* might be beneficial, since such enzymes degrade
the milk proteins differently. This might add to the
development of diary products with improved or new
characteristics. Neutral protease variants were selected
that, as the result of single amino acid substitutions,
20  behave differently towards protein substrates. Firstly, a NP
variant was constructed that had residue Gln120 substituted
by Cys, using site-directed mutagenesis. This residue is
located near the entrance of the active site and it was
anticipated that, by changing the character of this residue,
25  penetrating of substrates would be affected. The mutant was
constructed using a synthetic oligonucleotide (Table 2),
essentially according to Stanssens *et al.* (1989). The
mutated gene was expressed in *B. subtilis* and the gene
product was purified (van den Burg *et al.,* 1989) and
30  characterized. A second NP variant was constructed in which
Phe134 was substituted by Leu. Inspection of the 3-D model
of NP suggested that this particular residue might be
involved in substrate binding. Changing the size of this
residue could have effect on the substrate specificity of
35  the NP. The substitution was accomplished with a synthetic
oligonucleotide (Table 2) and the gene was reintroduced and
expressed in *B. subtilis*. Thirdly, Gly136 was substituted by

Ala. Since residue 136 is located in the α-helix connecting the N- and C-terminal domains, it was anticipated that by introduction of a larger side chain the hinge-bending, being the observed motion between the domains, could be decreased.

5       The substitution was constructed as described above (see Table 2 for oligonucleotide used).

The NP variants were produced by *B. subtilis* and purified from the culture supernatants by affinity chromatography (van den Burg *et al.*, 1989). Purification

10      yields and purity were analyzed by SDS-PAGE. Yields and purities were similar to the wild-type except for the variant Gln120Cys. In the latter case high molecular weight aggregates, caused by intermolecular disulfide bond formation, were visible during SDS-PAGE in the absence of

15      reducing agent. However, it was shown that this aggregation was caused by boiling the samples prior to electrophoresis, since it was absent in case the samples were heated at lower temperatures.

Specificic activities of the variants were determined

20      using casein and the synthetic substrates FAGLA (N-(3-[2-furyl]acryloloyl)-Gly-Leu amide) and FAAFA (N-(3-[2-furyl]acryloloyl)-AlA-Phe amide) as substrates.

Substrate specificity was analysed and compared to the wildtype NP using β-casein as model substrate. Enzyme and

25      substrate were incubated at a ratio of $10^{-3}$ for 16 hours at 30°C. After separation of the acid soluble and insoluble peptides, the reaction products were analyzed using Reversed Phase-HPLC. As becomes evident from Figure 12, substrate specificity towards β-casein is effected by substituting

30      residues 120, 134 and 136. After introduction of the gene encoding these variants into the chromosome of *L. lactis*, using the procedure described in Example 1, contribution of these variants to the flavour development in dairy products can be tested. Furthermore, new dairy products might be the

35      result from the use of variants such as those discussed above. Neutrol protease production levels of the resulting

strains LB282, LB292 and LB297 were determined and fond to
be similar to those of LB212 and LB213 (Table 3).

EXAMPLE 4

5

*Construction of Saccharomyces strain and E.coli expressing
B. subtilis neutral protease variants with altered activity.*
      The gene encoding *B.subtilis* neutral protease was
cloned into *Escherischia coli* and the yeast shuttle vector
10    pLF1 with and without pre and/or pro coding sequences, The
gene was placed under control of yeast regulatory sequences
(PKG gene). After transformation of these constructs into
*Saccharomyces cerevisiae* the presence of active protease was
determined in intacellular and extracellular fractions. In
15    the case that both pre and pro sequences were present on the
gene that was expressed the product accumulated in the yeast
cytoplasm in an inactive form. This form turned out to be
the pre-pro-neutral protease precursor. Apparently the
export signals of this gene are not operable in
20    *S.cerevisiae*. Transport of the product over membranes is
necessary for the product to obtain the right conformation
of which the pro sequence can be cleaved off. The gene
without prepro sequences was therefore provided with the
yeast invertase export signals. The product was now excreted
25    into the medium. However, an active product was still not
obtained, because the neutral protease was glycosylated,
which is normally not the case for neutral proteases. For
expression of an active product using heterologous export
signals in *S.cerevisiae* it is therefore necessary to prevent
30    glycosylation, for instance by altering the glycosylation
signals. *E.coli* does express and excrete an active neutral
protease.

EXAMPLE 5

*Production of laboratory scale cheeses using bacteria*
*provided with neutral protease activity according to the*
5  *invention ((Testing L. lactis LB212, LB213 and LB214 in*
*cheese production).*

Two Gouda type rindless cheeses of 175g were prepared
for each different set of parameters of the experiment as
given in table 4. To the milk of the reference experiment
10  Calcium Chloride and rennet was added together with 0.6% of
starter culture Bos, an often used production start of
culture. After coagulation and cutting 50% of the whey was
removed and 30% rinsing water was added. After 50
min.stirring the curd was separated from the whey and
15  subsequently pressed in cheesemoulds. A further reference
experiment included the addition of 0.1 to 0.4% calculated
on the milk of the mother microorganism culture besides the
starter culture. The experiments employing neutral protease
activity according to the invention comprised the same
20  addition of a microorganism of the mother culture, but than
genetically modified as disclosed in table 5. The cheese
production process was the same throughout the set of
experiments. The resulting cheeses were put in brine for
several hours and subsequently wrapped in Cryovac foil. They
25  were allowed to ripen at a temperature of 7 degrees Celsius.
After three, respectively 12 weeks of ripening the cheeses
were analysed for composition, i.e. moisture content, fat
content, salt content and pH. Nitrogen analyses of the
cheeses (total N), aqueous extracts (soluble N) of the
30  cheeses and cheeses after aqueous extraction (amino N)were
also performed. The results are given in table 4.

FIGURE LEGENDS

Figure 1.a. Alignment of the primary sequences of neutral
proteases from *Bacillius thermoproteolyticus* (1),

5   *B.stearthermophilus* (2), *B. cereus* (3), *B. Subtilis* (4) and
*B. amyloliquefaciens* (5).
Figure 1.b. Nucleotide sequence of the mature part of the
neutral protease gene from *B. subtilis* and the drerived
amino acid sequence. Positions and character of amino acid

10  substitutions are shown in bold.
Figure 2. Three-dimensional models of thermolysin (A) and
*B. subtilis* neutral protease (B). Active site of thermolysin
is indicated by an arrow. The dotted circle represents the
zinc ion, the filled-in circles indicate calcium ions.

15  Figure 3. Plasmid pGS1, containing the wild-type
*B. subtilis* neutral protease.
Figure 4. Plasmid pORI28
Figure 5. Nucleotide sequences of regulatory DNA fragments
P23, P32 and P44. Positions of the oligonucleotides used to

20  amplify the promotor sequences by PCR are shown.
Figure 6. Plasmid pINT51
Figure 7. Intermediates of the procedure used to integrate
neutral protease gene variants in *Lactococcus lactis*.(A)
part of the genome of *L. lactis* LB250.(B) and (C) represent

25  the two possible integrations obtained after integration of
the pINT51 derivate containing the wild-type neutral
protease gene and P32.(D) strain LB213, obtained after
excission of the pINT51 sequences.
Figure 8. Plasmids pCR10, pCR12, pCR13 and pCR14.

30  Figure 9. Plasmids pCR112, pCR113 and pCR114.
Figure 10. Southern hybridization analysis of the
construction of LB213. The *B.subtilis* neutral protease gene
was used as a probe. In all lanes EcoRV digested chromosomal
DNA was loaded from respectively, (1) LB250; (2) strain

35  LB113, wherein pCR113 has been integrated (see Figure 7.C);
(3) and (4) strain from which the complete pCR113 sequence
was removed by homologous recombination, yielding LB250

again; (5) and (6) strain LB213, obtained arter excission of the pINT51 sequences (see also Figure 7.D).

*Figure 11*. Growth curvesof wild-type *L.lactis* strains and variants expressing the *B.subtilis npr* gene (A), and growth and neutral protease production of strains LB212 and LB213 (B).

*Figure 12*.   Reversed phase-HPLC chromatography patterns as obtained by chromatography of β-casein preparations digested with wild-type neutral protease (A) and variants; Gln120Cys (B), Phe133Leu (C), Gly135Cys (D). Peptides were eluted with a lineair gradient of acetonitril in Milli Q water from 5 to 60% in 30 minutes.

## REFERENCES

Alber, T. 1989. Annu. Rev. Biochem. 58: 765-798.

Braxton, S., and Wells, J.A. 1992. Incorporation of a

5    stabilizing $Ca^{2+}$-binding loop into subtilisin BPN'. Biochem.
31:7796-7801.

Colman, P.M., Jansonius, J.N., and Matthews, B.W. 1972.
J. Mol. Biol. 70:701-724.

Corbett, R.J.T., and Roche, R.S. 1983. The unfolding

10   mechanism of thermolysin. Bioplymers 22:101-105.

Dahlquist, F.W., Long, J.W., and Bigbee, W.L. 1976. Role of
calcium in thermal stabilitu of thermolysin. Biochem.
15:1103-1111.

Drucker, H., and Borchers, S.L. 1971. The role of calcium in

15   thermolysin: effect on kinetic properties and autodigestion.
Arch. Biochem. Biophys. 147:242-248.

Dunn, B.M. (1989) in: Proteolytic Enzymes, a Practical
Approach, Beynon, R.J., and Bond, J.S. (eds) IRL Press,
Oxford, UK, pp. 57-82.

20   Eijsink et al. 1990. Prot. Engng. 4:99-104.

Eijsink, V.G.H. 1991. Engineering the thermostability of
Bacillus neutral proteases. Academic thesis. Groningen State
University.

Eijsink, V.G.H., Van den Burg, B., Vriend, G., Berendsen,

25   H.J.C., and Venema, G. 1991. Thermostability of Bacillus
subtilis neutral protease. Biochem. International 24:517-
525.

Eijsink, V.G.H., Vriend, G., Van der Vinne, B., Hazes, B.,
Van den Burg, B., and Venema, G. 1992. Effects of changing

30   the interaction between subdomains on the thermostability of
Bacillus neutral proteases. Proteins Struct. Funct. Genet.
14:224-236.

El Soda, M.A. 1993. The role of lactic acid bacteria in
accelerated cheese ripening. FEMS. Microbiol. Rev. 12:239-

35   252.

Imanaka, T., Shibazaki, M., and Takagi, M. 1986. A new way
of enhancing the stability of proteases. Nature 324:695-697.

Isowa, Y., Ohmori, M., Ichikawa, T., Mori, K., Nonaka, Y., Kihara, K., Oyami, K., Satoh, H., and Nishimura, S. 1979. The thermolysin-catalyzed condensation reactions of N-substituted aspartic and glutamic acids with phenylalanine alkyl esters. Tetrahedron letters 28:2611-2612.

Kester, W.R., and Matthews, B.W. 1977. J. Biol. Chem. 252:7704-7710.

Kok, J. 1991. in Genetics and molecular biology of Streptococci, Lactococci and Enterococci. ed. G.M. Dunny, P.P. Cleary, and L.L. McKay. 97-102.

Kubo, M., and Imanaka, T. 1988. Cloning and nucleotide sequence of the highly thermostable neutral protease gene from *Bacillus stearothermophilus*. J. Gen. Microbiol. 134:1883-1892.

Kubo, M., Mitsuda, Y., Takagi, M., and Imanaka, T. 1992. Alteration of specific activity and stability of thermostable neutral protease by site-directed mutagenesis. Appl. Environm. Microbiol. 58:3779-3783.

Leenhouts, K, and Venema, G. 1992. Molecular cloning and expression in *Lactococcus*. Med. Fac. Landbouww. Univ. Gent 2031-2043.

Lindgren, S.E., and W.J. Dobrogosz. 1990. Antagonistic activities of lactic acid bacteria in food and feed fermentations. FEMS Microbiol. Rev. 87:149-164.

Marquardt, R., Hilgenfield, R., and Keller, R. 1990. Proteasegen aus *bacillus thermoproteolyticus* rokko, Verfahren zu seiner Gwinnung und seine Verwending. European patent EP 90 11 6858/0 418 625 A1.

Matthews, B.W., Jansonius, J.N., Colman, P.M., Schoenbron, B.P., and Duporque, D. 1972a. Nature 238:37-40.

Matthews, B.W., Colman, P.M., Jansonius, J.N., Titani, K., Walsh, K.A., and Neurath, H. 1972b. Nature 238:41-43.

Matthews, B.W. 1988. Structural basis of the action of thermolysin and related zinc peptidases. Acc. Chem. Res. 21:333-340.

McGarry, A., Law, J., Coffey, A., Daly, C., Fox, P.F., and Fitzgerald, G.F. 1994. Effect of genetically modifying the

lactococcal proteolytic system on ripening and flavor
development in Cheddar cheese. Appl. Environ. Microbiol.
60:4226-4233.

McKay, L.L., and K.A. Baldwin. 1990. Applications for
5  biotechnology: present and future improvements in lactic
acid bacteria. FEMS Microbiol. Rev. 87:3-14.

Olson, N.F. 1990. The impact of lactic acid bacteria on
cheese flavor. FEMS Microbiol. Rev. 87:131-148.

Stanssens, P., Opsomer, C., Mckown, Y.M., Kramer, W.,
10  Zabeau, M., and Fritz, H-.J. 1989. Efficient oligonucleotide
directed construction of mutations in expression vectors by
the gapped duplex DNA method using alternating selectable
markers. Nucl. Acids Res. 17:4441-4454.

Stark et al. 1992. The structure of neutral protease from
15  *Bacillus cereus* at 0.2 nm resolution. Eur. J. Biochem.
207:781-791.

Towdin, H. and Gordon, J. (1984) J. Immunol. Meth. 72, 313.

Pauptit, R.A., Karlsson, R., Picot, D., Jenkins, J.A>,
Niklaus-Reimer, A.-S., and Jansonius, J.N. 1988.J. Mol.
20  Biol. 199:525-537.

Pjura, P.E., Matsumura, M., Wozniak, J.A., and Matthews,
B.W. 1990. Biochem. 29:2592-2598.

Roche, R.S., and Voordouw, G. 1978. The structural and
functional roles of metal ions in thermolysin. CRC. Crit.
25  Rev. Biochem. 5:1-23.

Takagi, M., Imanaka, T., and Aiba,.S. 1985. Nucleotide
squence and promotor region for the neutral protease gene
from *Bacillus stearothermophilus*. J. Bacteriol. 163:824-831.

Toma, S., Campognoli, S., De Gregoriis, E., Gioanna, R.,
30  Margarit, I., Zamai, M., and Grandi, G. 1989.
Prot. Engng. 2:359-364..

Toma, S., Campagnoli, S., Margarit, I., Gianna, R., Grandi,
G., Bolognesi, M., De Filippis, V., and Fontana, A. 1991.
Grafting of a calcium-binding loop of thermolysin to
35  *Bacillus subtilis* neutral protease. Biochem. 30:97-106.

Tronrud, D.E., Monzingo, A.F., and Matthews, B.W. 1986.
Eur. J. Biochem. 157:261-268.

31

Van de Guchte, M., Kodde, J., Van der Vossen, J., Kok, J., and Venema, G. 1990. Heterologous gene expression in *Lactococcus lactis subsp. lactis*. Synthesis, secretion and processing of the *Bacillus subtilis* neutral protease. Appl.

5  Environ. Microbiol. 56:2606-2611.

Van den Burg, B., Enequist, H.G., Van der Haar, M.E., Eijsink, V.G.H., Stulp, B.K., and Venema, G. 1991. A highly thermostable neutral protease from *Bacillus caldolyticus*, cloning and expression of the gene in *Bacillus subtilis* and

10  characterization of the gene product. J. Bacteriol. 173:4107-4115.

Vriend, G., and Eijsink, V.G.H. 1993. Prediction and analysis of structure, stability and unfolding of *Bacillus* neutral proteases. J. Computer-Aided Mol. Design 7:367-396.

15  Wetzel, R. 1987. Trends Biochem. Sci. 12:478-482.

32

## Table 1

*Thermal stabilities of B. subtilis neutral protease variants*

| neutral protease | T50 (°C) | δT50 |
|---|---|---|
| wild-type | 58 | 0 |
| Leu300Ala | 48 | -10 |
| Leu300Ile | 50 | - 8 |
| Leu300Asn | 45 | -13 |
| Leu300del | 42 | -16 |
| Val102Cys/Gln120Cys | 52 | - 6 |
| Glu186Cys/Ala194Cys | 48 | -10 |

T50 is the temperature at which 50% of the activity is lost
in 30 minutes.

Table 2. Synthetic oligonucleotides used for PCR

| name | sequence | characteristics |
|---|---|---|
| NP-5' | AAAACGCGTCGACCACGTGGGTTTAGGTAAG | start *npr* gene, MluI, SalI, PmlI |
| NP-3' | CGCGGATCCGTCGACATTACAATCCAACAGCATTCCAGGC | stop *npr* gene, BamHI, SalI |
| P23-5' | TTCGAACTGCAGACGCGTGATCGCATCATTTTC | "start" P23, BstBI, PstI, MluI |
| P23-3' | AAAGCGATGCATATATTTGGCCTCCC | "end" P23, NsiI |
| P32-5' | TTCGAACTGCAGACGCGTCCGGTCCTCGGG | "start" P32, BstBI, PstI, MluI |
| P32-3' | AAAGCGATGCATTCAAAATTCCTCC | "end" P32, NsiI |
| P44-5' | TTGGAAACGCGTCAGAACGATGAAAAAAG | "start" P44, BstBI, MluI |
| P44-3' | AAAGCTTCGAAAAGCGACTCCTTTC | "end" P44, HindIII, BstBI |
| Leu300Ala | CTAATATCACGCTCCAACAGC | replaces Leu300 by Ala, SspI- |
| Leu300Ile | CTAATATTAAATTCCAACAGCGTTCCAGGC | replaces Leu300 by Ile, BsmI- |
| Leu300Asn | CTAATATTAATTTCCAACAGCATTCC | replaces Leu300 by Asn, AsnI+ |
| Leu300del | CTAATATTACAATCCAACAGCGTTCCAGGC | deletes Leu300, BsmI- |
| Val102Cys | GTGCACGGAAGAACAGATTTTACTGCC | replaces Val102 by Cys, SnoI+ |
| Gln120Cys | CCGTAAATCATGCAGTCTCCGGTCC | replaces Gln120 by Cys, AvaII+ |
| Glu186Cys | CCGTAATGTGGCAACCGATATCCC | replaces Glu186 by Cys, EcoRV+ |
| Ala194Cys | GGACAAGCTTCGAAGACAAGGCTGGC | replaces Ala194 by Cys, HindIII+ |
| Phe134Leu | TAATGAGCCGGAGAACGGAGAGAA | replaces Phe134 by Leu |
| Gly136Ala | CACATCTAATGACGCGGAAAGCGG | replaces Gly136 by Ala |

Table 3. Extracullar neutral protease activities of different promotor contructs and different specificity variants. Activity was determined using the azo-casein (A440) assay.

| STRAIN | Absorbance at 440 nm |
|---|---|
| | |
| LB250 (Npr-) | 0 |
| LB212 (P23-Npr) | 0.325 |
| LB213 (P32-Npr) | 0.352 |
| LB214 (P44-Npr) | 0.022 |
| LB282 (P32-Npr120Cys) | 0.221 |
| LB292 (P32-Npr134Phe) | 0.191 |
| LB297 (P32-Npr136Ala) | 0.168 |

Table 4

| cheese starter | pH | | moisture (%) pasteurized cheese | salt | TH | | SH/TH | | AN/TH | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 3 weeks | 12 weeks | mean | mean | 3 weeks | 12 weeks | 3 weeks | 12 weeks | 3 weeks | 12 weeks |
| reference 1 | 5.41 | 5.56 | 40.8 | 3.1 | 3.8 | 3.9 | 4.4 | 10.7 | 3.2 | 9.1 |
| reference 2 | 5.35 | 5.46 | 42.1 | 3.5 | 3.8 | 3.0 | 4.5 | 10.7 | 3.5 | 9.1 |
| reference 3 | 5.36 | 5.45 | 41.0 | 3.1 | 3.9 | 3.0 | 4.0 | 11.4 | 3.0 | 8.5 |
| reference 4 | 5.37 | 5.40 | 42.1 | 3.7 | 3.6 | 3.0 | 4.4 | 12.0 | 3.5 | 9.6 |
| reference 5 | 5.29 | 5.40 | 42.5 | 3.5 | 3.7 | 3.8 | 4.5 | 11.8 | 3.2 | 8.8 |
| LB250 0.4% | 5.31 | 5.42 | 43.6 | 3.8 | 3.7 | 3.7 | 4.6 | 10.8 | 3.8 | 8.8 |
| LB250 0.1% | 5.22 | 5.39 | 42.6 | 3.4 | 3.7 | 3.7 | 4.4 | 9.4 | 3.2 | 8.8 |
| LB250 0.4% | 5.38 | 5.53 | 42.2 | 3.4 | 3.8 | 3.8 | 4.5 | 9.0 | 3.4 | 7.5 |
| LB212 0.4% | 5.33 | 5.44 | 42.0 | 3.1 | 4.1 | 3.8 | 8.5 | 24.7 | 5.7 | 20.1 |
| LB212 0.7% | 5.30 | 5.41 | 41.8 | 3.2 | 3.8 | 3.7 | 4.3 | 14.6 | 3.4 | 11.9 |
| LB212 0.4% | 5.42 | 5.65 | 41.0 | 3.0 | 3.8 | 4.0 | 5.8 | 17.7 | 4.3 | 14.3 |
| LB212 0.1% | 5.37 | 5.57 | 40.8 | 3.0 | 3.8 | 3.9 | 4.4 | 14.4 | 3.2 | 11.8 |
| LB213 0.4% | 5.29 | 5.55 | 43.5 | 3.6 | 3.6 | 3.8 | 7.7 | 17.9 | 5.4 | 14.0 |
| LB213 0.1% | 5.28 | 5.50 | 43.7 | 3.7 | 3.7 | 3.6 | 5.4 | 12.1 | 4.3 | 10.0 |
| LB214 0.4% | 5.31 | 5.47 | 42.2 | 3.2 | 3.8 | 3.8 | 3.9 | 11.8 | 3.1 | 9.8 |
| LB214 0.1% | 5.21 | 5.26 | 41.9 | 3.3 | 3.7 | 3.6 | 4.5 | 10.8 | 3.6 | 8.7 |
| LB215 0.4% | 5.36 | 5.52 | 43.6 | 3.6 | 3.7 | 3.7 | 4.5 | 11.5 | 4.0 | 0.8 |
| LB215 0.1% | 5.39 | 5.44 | 42.7 | 3.7 | 3.6 | 3.6 | 4.6 | 12.0 | 3.5 | 9.3 |

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## CLAIMS

1.   A method for carrying out a process of
biotransformation of a substrate whereby at least one lactic
acid bacterium comprising a gene encoding a neutral protease
having an altered resulting activity is used.

5    2.   A method according to claim 1 whereby at least one
neutral protease has an altered stability or specificity.

3.   A method according to claim 1 or 2, whereby the altered
resulting activity is accomplished by regulation of
expression of at least one neutral protease gene.

10   4.   A method according to any one of claims 1-3, whereby
the biotransformation process is fermentation of a food
product.

5.   A method according to claim 4 whereby the substrate is
milk or a milk derivative.

15   6.   A method according to claim 5 whereby the
biotransformation process is a part of a cheese making
process.

7.   A method according to anyone of the aforegoing claims
wherein the gene encoding a neutral protease is derived from

20   a gene of the genus Bacillus.

8.   A method according to claim 7 whereby the gene is
derived from Bacillus subtilis.

9.   A method according to claim 8 whereby the gene
comprises at least one of the following mutations: mutation

25   of the codon encoding leucine at position 300;

               : deletion of the codon encoding leucine at
position 300;

               : mutation of the codons for residues 120, 134
and/or 136;

30             : mutation of the codons 186 and 194 to cysteine
residues; or

               : mutation of the codons 102 and 120 to cysteine
residues.

10. A method according to anyone of the aforegoing claims, whereby the lactic acid bacterium is a Lactococcus species.

11. A method according to anyone of the aforegoing claims whereby the gene encoding the neutral protease is integrated into the genome of the lactic acid bacterium without residual transfecting material.

12. A food product obtainable by a method according to any one of the aforegoing claims.

13. A food product according to claim 12 which is cheese.

Figure 1. A

```
                                                                    15
GCC GCC GCC ACT GGA AGC GGA ACA ACG CTA AAG GGC GCA ACT GTT
Ala Ala Ala Thr Gly Ser Gly Thr Thr Leu Lys Gly Ala Thr Val

                                                                    30
CCT TTG AAC ATC TCT TAT GAA GGC GGA AAA TAT GTT CTA AGA GAT
Pro Leu Asn Ile Ser Tyr Glu Gly Gly Lys Tyr Val Leu Arg Asp

                                                                    45
CTT TCA AAA CCA ACA GGC ACC CAA ATC ATC ACA TAT GAT TTG CAA
Leu Ser Lys Pro Thr Gly Thr Gln Ile Ile Thr Tyr Asp Leu Gln

                                                                    60
AAC AGA CAA AGC CGC CTT CCG GGC ACG CTT GTC TCA AGC ACA ACG
Asn Arg Gln Ser Arg Leu Pro Gly Thr Leu Val Ser Ser Thr Thr

                                                                    75
AAA ACA TTT ACA TCT TCA TCA CAG CGG GCA GCC GTT GAC GCA CAC
Lys Thr Phe Thr Ser Ser Ser Gln Arg Ala Ala Val Asp Ala His

                                                                    90
TAT AAC CTC GGT AAA GTG TAC GAT TAT TTT TAT TCA AAC TTT AAA
Tyr Asn Leu Gly Lys Val Tyr Asp Tyr Phe Tyr Ser Asn Phe Lys

                                                                    105
CGA AAC AGC TAT GAT AAC AAA GGC AGT AAA ATC GTT TCT TCC GTT
Arg Asn Ser Tyr Asp Asn Lys Gly Ser Lys Ile Val Ser Ser Val
                                                                    Cys
                                                                    120
CAC TAC GGC ACT CAA TAC AAT AAC GCT GCA TGG ACA GGA GAC CAG
His Tyr Gly Thr Gln Tyr Asn Asn Ala Ala Trp Thr Gly Asp Gln
                                                                    Cys
                                                                    135
ATG ATT TAC GGT GAT GGC GAC GGT TCA TTC TTC TCT CCG CTT TCC
MET Ile Tyr Gly Asp Gly Asp Gly Ser Phe Phe Ser Pro Leu Ser
                                                                    Phe
                                                                    150
GGC TCA TTA GAT GTG ACA GCG CAT GAA ATG ACA CAT GGC GTC ACC
Gly Ser Leu Asp Val Thr Ala His Glu MET Thr His Gly Val Thr
Ala
                                                                    165
CAA GAA ACA GCC AAC TTG ATT TAT GAA AAT CAG CCA GGT GCA TTA
Gln Glu Thr Ala Asn Leu Ile Tyr Glu Asn Gln Pro Gly Ala Leu

                                                                    180
AAC GAG TCT TTC TCT GAC GTA TTC GGG TAT TTT AAC GAT ACA GAA
Asn Glu Ser Phe Ser Asp Val Phe Gly Tyr Phe Asn Asp Thr Glu

                                                                    195
GAC TGG GAC ATC GGT GAA GAC ATT ACG GTC AGC CAG CCT GCT CTT
Asp Trp Asp Ile Gly Glu Asp Ile Thr Val Ser Gln Pro Ala Leu
                        Cys                                 Cys
                                                                    210
CGC AGC CTG TCC AAC CCT ACA AAA TAC AAC CAG CCT GAC AAT TAC
Arg Ser Leu Ser Asn Pro Thr Lys Tyr Asn Gln Pro Asp Asn Tyr

                                                                    225
GCC AAT TAC CGA AAC CTT CCA AAC ACA GAT GAA GGC GAT TAT GGC
Ala Asn Tyr Arg Asn Leu Pro Asn Thr Asp Glu Gly Asp Tyr Gly

                                                                    240
GGT GTA CAC ACA AAC AGC GGA ATT CCA AAC AAA GCC GCT TAC AAC
Gly Val His Thr Asn Ser Gly Ile Pro Asn Lys Ala Ala Tyr Asn
```

```
                                                      255
ACC ATC ACA AAA CTT GGT GTA TCT AAA TCA CAG CAA ATC TAT TAC
Thr Ile Thr Lys Leu Gly Val Ser Lys Ser Gln Gln Ile Tyr Tyr


                                                      270
CGT GCG TTA ACA ACG TAC CTC ACG CCT TCT TCC ACG TTC AAA GAT
Arg Ala Leu Thr Thr Tyr Leu Thr Pro Ser Ser Thr Phe Lys Asp

                                                      285
GCC AAG GCA GCT CTC ATT CAG TCT GCC CGT GAC CTC TAC GGC TCA
Ala Lys Ala Ala Leu Ile Gln Ser Ala Arg Asp Leu Tyr Gly Ser

                                                      300
ACT GAT GCC GCT AAA GTT GAA GCA GCC TGG AAT GCT GTT GGA TTG
Thr Asp Ala Ala Lys Val Glu Ala Ala Trp Asn Ala Val Gly Leu
                                          .                 Ala
                                                            Ile
                                                            Asn
                                                            del
```

Figure 1.B. Nucleotide sequence of the mature part the neutral protease
gene from *Bacillus subtilis* and the derived amino acid sequence. Positions
and character of amino acid substitutions are shown in bold.
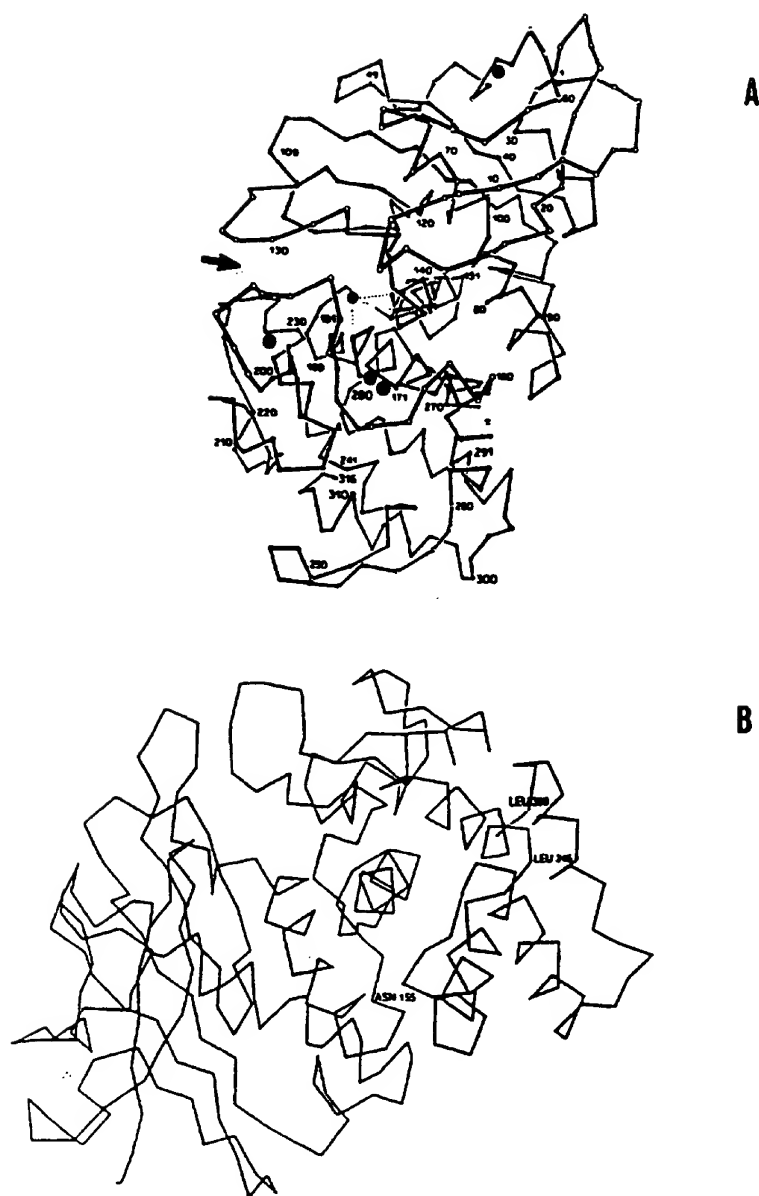
Figure 2. Three-dimensional models of thermolysin (A) and *Bacillus subtilis* neutral protease (B). Active site of thermolysin is indicated by an arrow. The dotted circle represents the zinc ion. the filled-in circles indicate calcium ions.
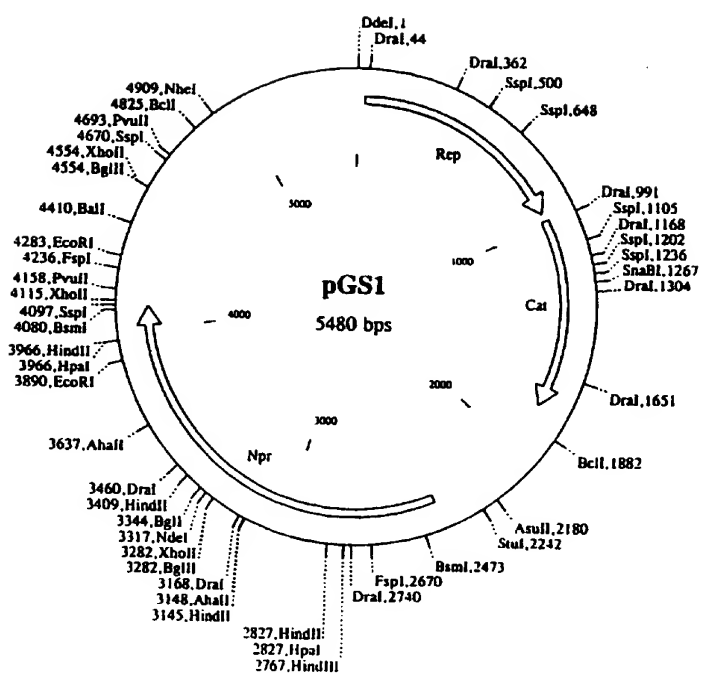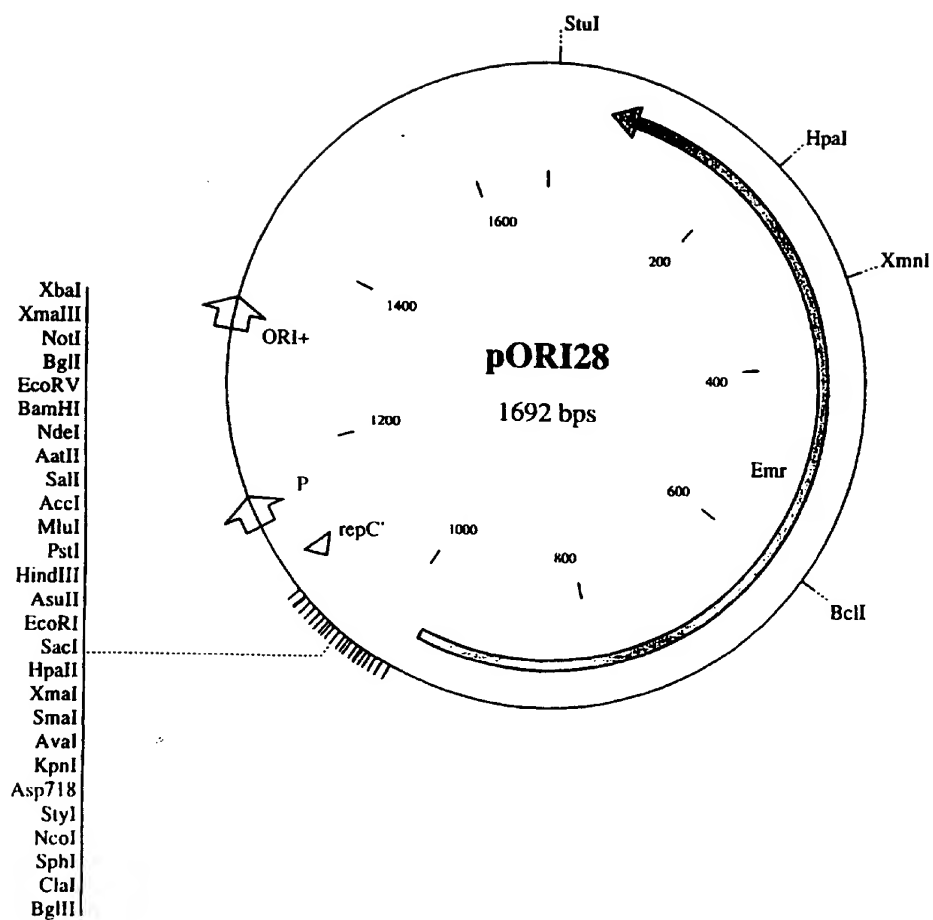
FIGURE 3.

FIGURE 4

**promoter 23**

=====>

TCG AAA AGC CCT GAC AAC CCT CGT TCC TAA AAA GGA ATA AGC GTT TGG TCA GTA AAT AAT

                                          -35                              -10
AGA AAT AAA AAA TCA GAC CTA AGA CTG ATG ACA AAA AGA GCA AAT TTT GAT AAA ATA GTA

                                    <=====
TTA GAA TTA AAT TAA AAA GGG AGG CCA AAT ATA ATG AAA AAT ATG AAT GAC AAT GAT GTT
                                          Met Lys Asn Met Asn Asp Asn Asp Val


**promoter 32**

=====>

AGA TTA ATA GTT TTA GCT ATT AAT CTT TTT TTA TTT TTA TTT AAG AAT GGC TTA ATA AAG

                                          -35                              -10
CGG TTA CTT TGG ATT TTT GTG AGC TTG GAC TAG AAA AAA ACT TCA CAA AAT GCT ATA CTA

                                    <=====
GGT AGC TTA AAA AAT ATT CGG AGG AAT TTT GAA ATG GCA ATC GTT TCA GCA GAA AAA TTC
                                          Met Ala Ile Val Ser Ala Glu Lys Phe


**promoter 44**

=====>

AAC AAT TGT AAC CCA TAC AGG AGA AGG GAC GAT AGC AAT TTT TTC AAT AAG TAG ACA AAG

                                                       -35
TAG AGA ATA ATT TAA TAA AAA ACT GAA AAA ATC ACA GCT AAA CTC TTG TTT TAC TTG ATT

          -10
TTA TGT TAA AAT AAT TAA TGA GTG TAA TTG TAT ATA AAA TTA TCT GTA CAC TTA CCT AAT

                                                    <====
TTA TTA AAA AAA AAT ATG AAT CGT GAT GTG TGA GGG AAA GGA GTC GCT TTT ATG GCC AAA
                                                              Met Ala Lys


**Figure 5.** Nucleotide sequences of the regulatory DNA fragments P23, P32 and P44. Positions of the oligonucleotides used to amplify the promoter sequences by PCR are shown by arrows.
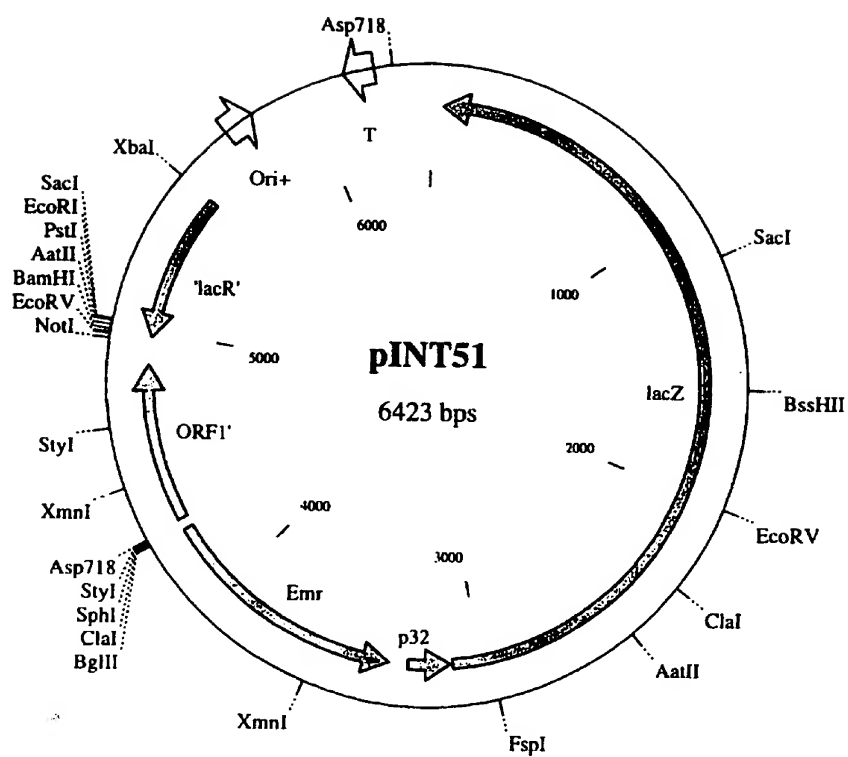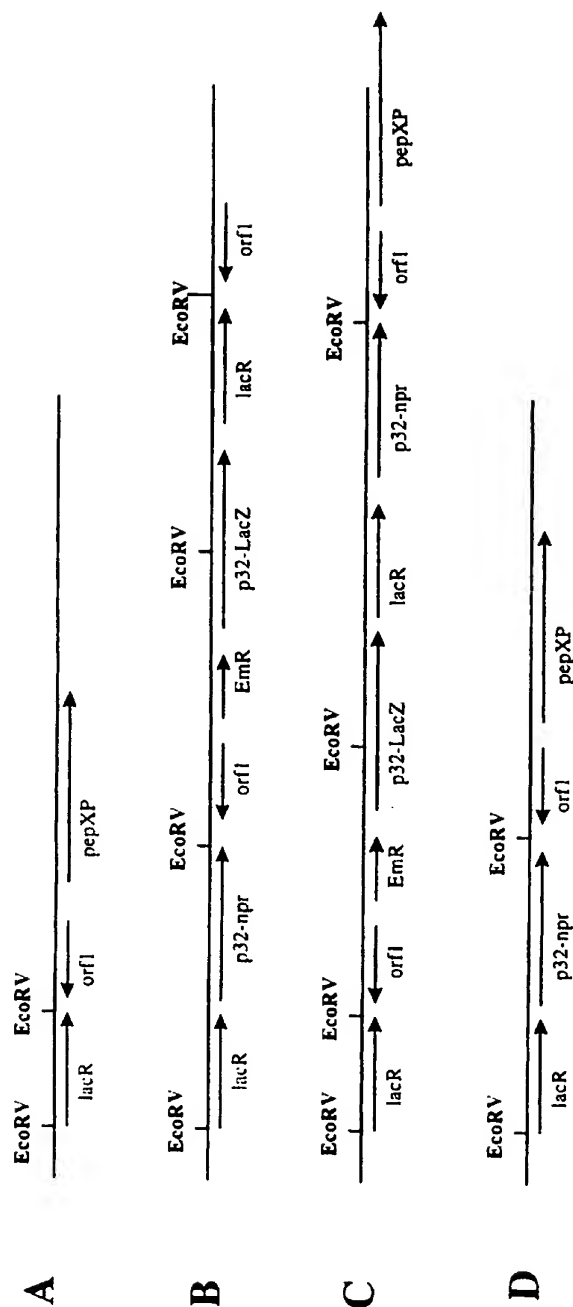

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FIGURE 6

**Figure 7.** Intermediates of the procedure used to integrate neutral protease gene variants in *Lactococcus lactis*. (A) part of the genome of *L. lactis* LB250. (B) and (C) represent the two possible integrations obtained after integration of the pINT51 derivate containing the wild-type neutral protease gene and p32. (D) strain LB213, obtained after excission of the pINT51 sequences.

MluI
PstI
HindIII
AsuII
EcoRI
SacI
HpaII
XmaI
SmaI
AvaI
KpnI
Asp718
StyI
NcoI
SphI
ClaI
BglII

HindIII
StyI
HpaI

BclI

Emr

3000

500

XmnI

BglII

HpaI

**pCR10**

3261 bps

npr

BglI
HpaII

2500

1000

2000

1500

StuI

HpaII

ORI+

repC'

EcoRI

BamHI
EcoRV
BglI
NotI
XmaIII
XbaI

HpaI
StyI

FIGURE 8.A

MluI
PstI
HindIII
AsuII
EcoRI
SacI
HpaII
XmaI
SmaI
AvaI
KpnI
Asp718
StyI
NcoI
SphI
ClaI
BglII

HindIII
StyI
HpaI

EcoRV

P23-PCR

3500

500

'npr

**pCR12**

**3768 bps**

3000

1000

BglII
BglI
HpaII

HpaII

BclI

Emr

2500

EcoRI

2000

1500

Xmnl

HpaI

repC'
P

EcoRI

HpaI
StyI

ORI+

BamHI
EcoRV
BglI
NotI
XmaIII
XbaI

StuI

FIGURE 8.B

FIGURE 8.C

MluI
PstI
HindIII
AsuII
EcoRI
SacI
HpaII
XmaI
SmaI
AvaI
KpnI
Asp718
StyI
NcoI
SphI
ClaI
BglII

XbaI
AvaI

PstI

Bcll

P44-PCR

AsuII

Emr          3500

500

HindIII
StyI
Hpal

XmnI

HpaI

pCR14

3916 bps

1000

StuI          3000

2500          1500          'npr

2000

BglII
BgII
HpaII

ORI+

P  repC'

HpaII

XbaI
XmaIII
NotI
BglI
EcoRV
BamHI

StyI          EcoRI
HpaI
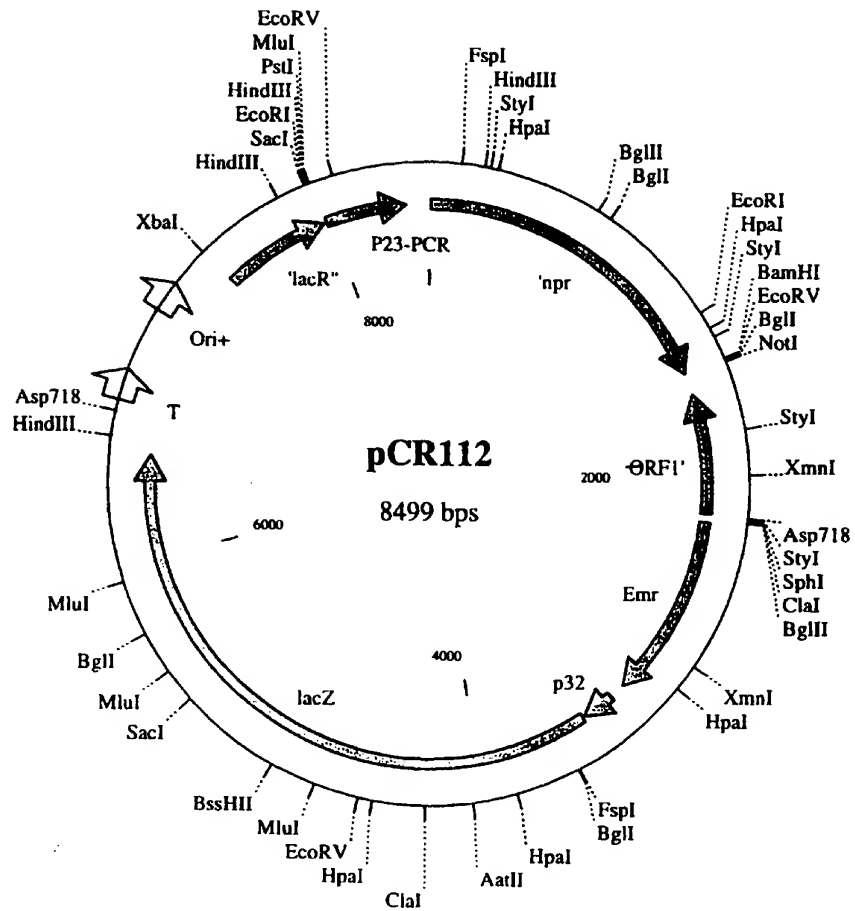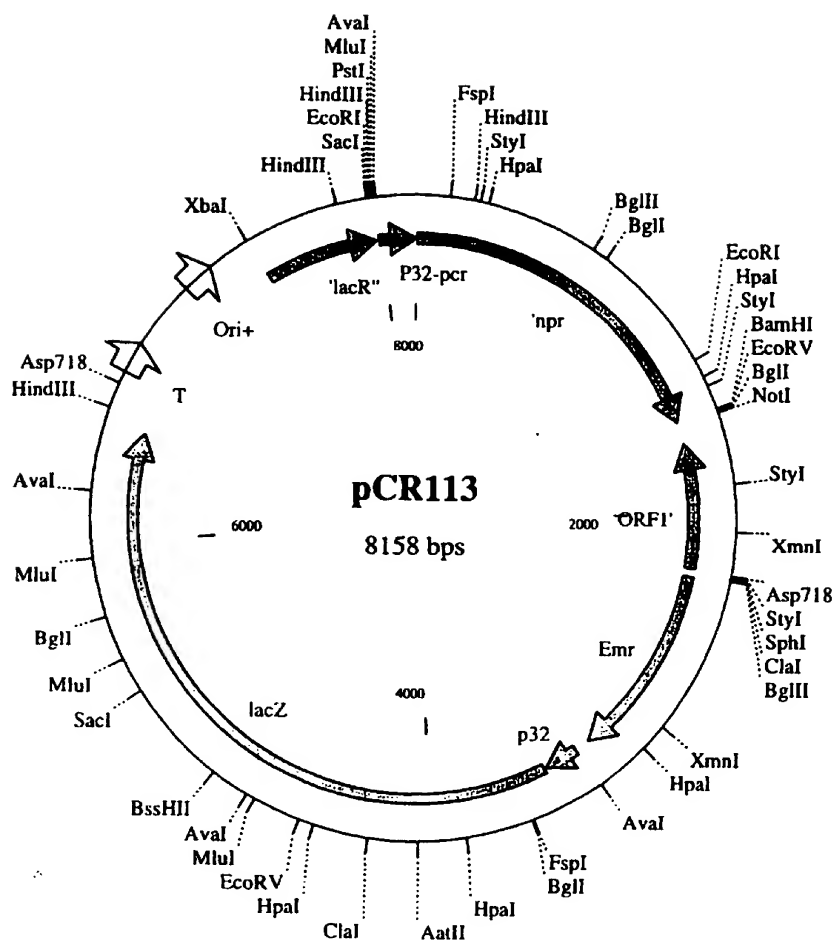
FIGURE 8.D
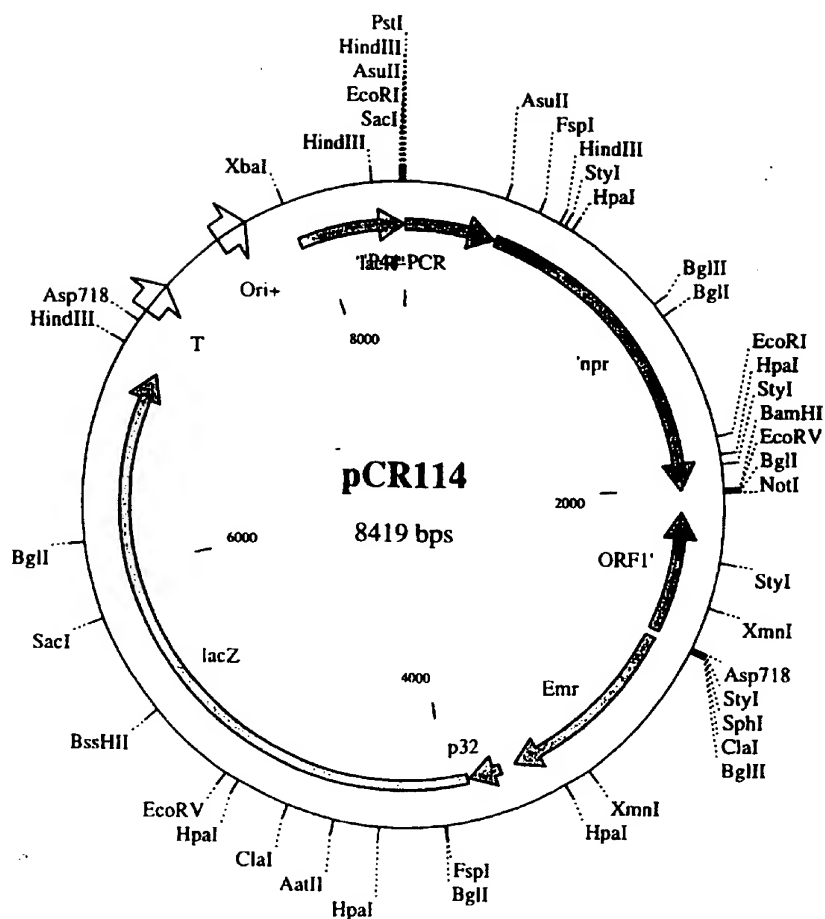
FIGURE 9.A

FIGURE 9.B

FIGURE 9.C

SUBSTITUTE SHEET (RULE 26)

FIGURE 10

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### Growth of wild-type and Npr strain: M17



### Growth and Npr production of LB212 and LB213
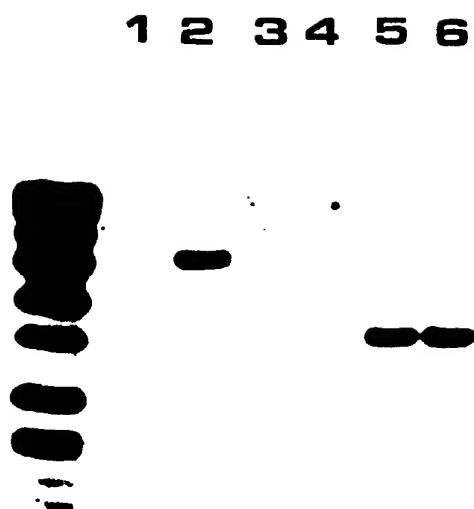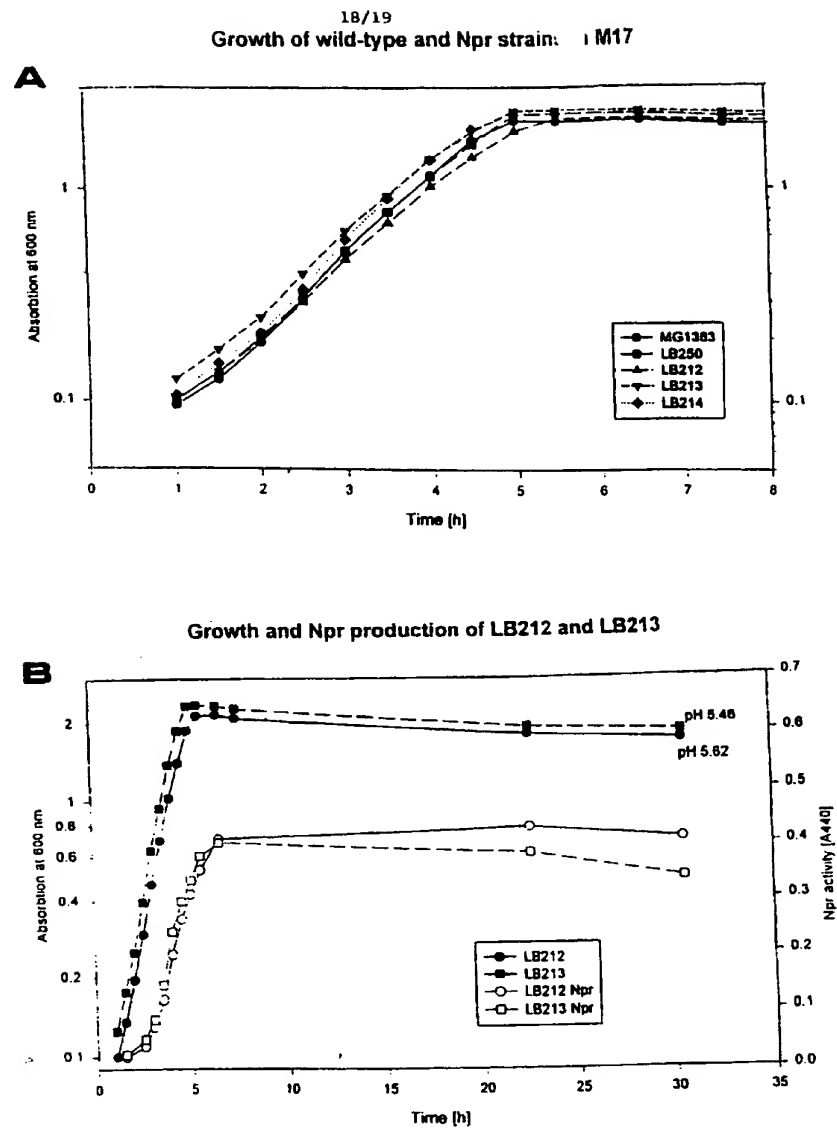


FIGURE 11

SUBSTITUTE SHEET (RULE 26)
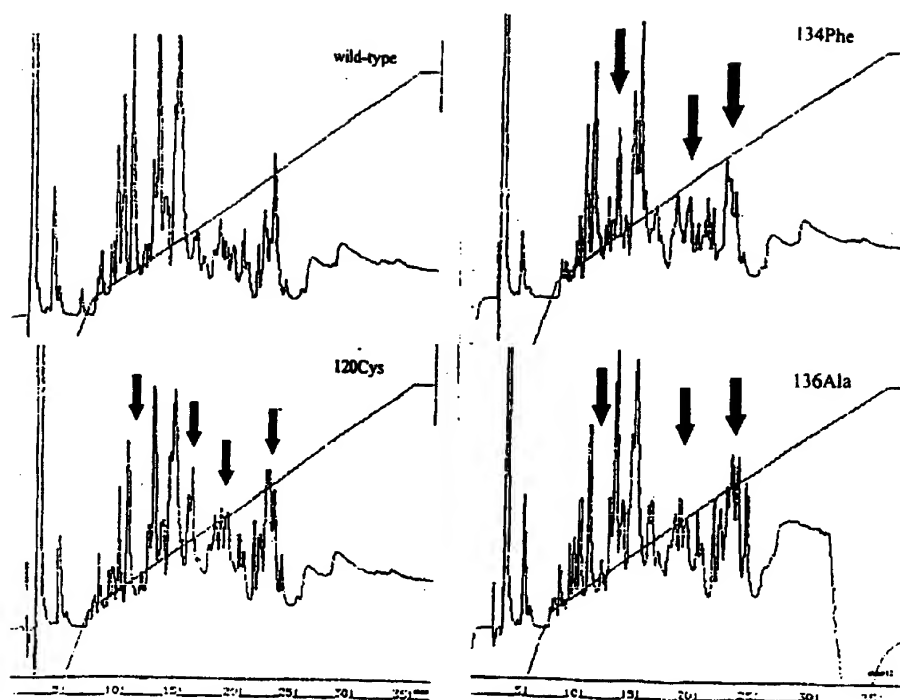
Figure 12. Reversed Phase-HPLC chromatography patterns as obtained by chromatography of β-casein preparations digested with wild-type neutral protease (A), and variants; Gln120Cys (B), Leu134Phe (C) and Gly136Ala (D). Peptides were eluted with a linear gradient of acetonitril in Milli Q water from 5 to 60 % in 30 minutes.

# INTERNATIONAL SEARCH REPORT

**A. CLASSIFICATION OF SUBJECT MATTER**
IPC 6    A23C19/032    C12N15/57    C12N15/75

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
IPC 6    A23C    C12M    C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | JOURNAL OF DAIRY SCIENCE, vol. 76, 1993, CHAPAIGN, ILLINOIS US, pages 2133-2144, XP000606357 G. VENEMA: "Molecular biology and genetic modification of lactococci" see page 2140, column 2 - page 2142, column 1 | 1,4-8, 12,13 |
| X | APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 56, no. 9, 1990, pages 2606-2611, XP000603149 M. VAN DE GUCHTE: "Heterologous gene expression in Lactococcus lactis subsp. lactis" cited in the application see page 2606, column 1 see page 2610 | 1,3-8, 11-13 |

-/--

| X | Further documents are listed in the continuation of box C. | | Patent family members are listed in annex. |

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 3 July 1997 | 18. 07. 97 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016 | Desmedt, G |

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 60, no. 12, 1994, pages 4226-4233, XP000603247 A. MCGARRY: "Effect of genetically modyfying the lactococcal proteolytic system on ripening and flavor development in cheddar cheese" cited in the application see page 4227, column 2 see page 4230 - page 4232 --- | 3-8,12, 13 |
| X | JOURNAL OF DAIRY SCIENCE, vol. 77, no. 8, 1994, CHAPAIGN, ILLINOIS US, pages 2150-2159, XP000468028 H. RIEPE: "Oversecration of the neutral protease from Bacillus subtilis in Lactococcus lactis spp. lactis JF254" see page 2150, column 2 - page 2151 see page 2153 see page 2158 --- | 1,2,4-8, 12,13 |
| X | JOURNAL OF DAIRY SCIENCE, vol. 76, no. 7, 1993, CHAPAIGN, ILLINOIS US, pages 2056-2064, XP002034378 J. KOK: "Genetics of proteolytic enzymes of lactococci and their role in cheese flavour development" see page 2057, column 2 - page 2058, column 2 --- | 1,2,4-8, 10,12,13 |
| A | JOURNAL OF CHEMICAL TECHNOLOGY AND BIOTECHNOLOGY, vol. 58, no. 2, 1993, OXFORD  GB, pages 195-199, XP000388270 G. FITZGERALD: "Molecular manipulations of Lactococcus starter cultures for food fermentations" see page 197 --- | 1-13 |
| A | JOURNAL OF DAIRY SCIENCE, vol. 76, no. 9, 1993, CHAPAIGN, ILLINOIS US, pages 2455-2467, XP000396656 J. LAW:  "The contribution of Lactococcal starter proteinases to proteolysis in cheddar cheese" see page 2455 - page 2456 --- | 1 |

-/--

1

**C.(Continuation)  DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | VOEDINGSMIDDELEN TECHNOLOGIE, vol. 20, no. 1, 1987, ZEIST  NL, pages 24-27, XP002015038<br>A. SIMONS:  "Genetisch onderzoek van melkzuurbacterien"<br>see page 25, column 3 - page 26, column 2<br>----- | 1 |

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)